Reduction of Intrinsic Steroid-Induced Apoptosis in the Development of Glucocorticoid Insensitivity

Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in the undergraduate colleges of The Ohio State University

By

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Abstract

Glucocorticoids (GCs) are involved in many psychological, cellular and molecular mechanisms in humans, and are vital to the preservation of stress-related homeostasis through their regulation of metabolic and immune functions. Alterations in tissue sensitivity to GCs define vulnerability to numerous diseases, including depression, diabetes, and chronic obstructive airways disease. Using an established murine model of social disruption stress (SDR), in which a male mouse is repeatedly defeated by an aggressor, we have previously shown that splenocytes of SDR-treated mice are less sensitive to the anti-inflammatory effects of GCs and resist GC-induced apoptosis. Although GC insensitivity has been repeatedly observed in SDR-treated mice, the mechanism by which it develops has not yet been fully elucidated. The present study aimed to characterize the resistance of GC-insensitive splenocytes to apoptosis by investigating the role of caspase-3 activity in splenocytes of stressed mice. The gene expression levels of CASP3 and CASP9 were also measured because of their direct relationship with caspase-3 activation in the intrinsic apoptotic pathway. Interestingly, capsase-3 activity was found to be elevated in the splenocytes of SDR- treated mice. In addition, both CASP3 and CASP9 expression was increased in SDR- treated mice. Taken together, the results demonstrate that caspase-3 is fully expressed and active in socially stressed mice and that resistance to glucocorticoid-induced apoptosis may be caused by apoptosis dysregulation downstream of caspase-3.

1. Introduction

1.1 Stress & Glucocorticoids

Stress has been implicated in the progression of many inflammatory diseases, yet definitive mechanisms by which it contributes to disease are lacking. Social stress, specifically, is associated with changes in immune function and behavior through the neuroendocrine activation and release of catecholamines and glucocorticoids (GCs). Previous work has shown repeated social defeat leads to the development of anxiety-like behavior, such as defensive burrowing, and the alteration of immune response to viral and bacterial challenges (Kinsey et al., 2007; Bailey et al., 2009; Mays et al., 2010). Social disruption stress (SDR), an animal model that subjects mice to repeated defeat in their home cage by an aggressive intruder, has been used to investigate the effects of repeated social defeat (Stark et al., 2001).

After six consecutive daily 2 h rounds of SDR, defeated mice show a significant increase in systemic corticosterone, a glucocorticoid hormone (Avitsur et al., 2001). The increased availability of corticosterone within the organism's tissues and blood can lead to alteration in the physiological responsiveness of peripheral myeloid cells to the glucocorticoid (Stark et al., 2001; Raison and Miller, 2003). Such alteration in tissue response is associated with vulnerability to numerous diseases, including depression, diabetes, and Chronic Obstructive Pulmonary Disease (COPD) (Barnes et al., 2009). Social stress impairs the capacity of GCs to terminate inflammatory response following immune challenge, but the mechanism is not well defined (Sheridan et al., 2000). SDR also promotes the trafficking of glucocorticoid resistant peripheral myeloid cells to the

spleen (Engler et al., 2004) and these resistant cells show increased production of inflammatory cytokines, such as interleukin-6 (IL-6), following Lipopolysaccharide (LPS) stimulation (Stark et al., 2002).

1.2 Caspases and Glucocorticoid-Induced Apoptosis

Apoptosis, or programmed cell death, like cellular proliferation and differentiation, is vital for the maintenance of an organism's homeostasis. The apoptotic process is characterized by cytoplasmic shrinkage, membrane blebbing, and nuclear and DNA fragmentation. Caspases, a family of cysteine proteases, are responsible for the execution of apoptosis in most eukaryotes (Cryns, 1998). With respect to apoptosis, caspases are classified as either "initiator" caspases or "executioner" caspases. Initiators, such as caspase-9, are defined by long pro-domains characterized protein-protein interfaction motifs that allow interaction with upstream adaptor molecules (Degterev et al., 2003). On the other hand, "executioner," or effector caspases, such as caspase-3, are characterized by short pro-domains and perform the downstream apoptosis steps by cleaving key cellular structures and halting essential cellular signaling pathways. Effectors are generally regulated by the upstream initiator caspases.

Caspase activation occurs in two distinct pathways, the extrinsic (death receptor pathway) or the intrinsic (mitochondrial pathway). In the extrinsic pathway, cell surface receptors, termed death receptors, bind to specific activate ligands and recruit downstream apoptotic proteins (Gupta, 2003). The intrinsic pathway, which is the dominant pathway in glucocorticoid-induced apoptosis, is stimulated by

chemotherapeutic agents and UV radiation. GCs stimulate the proapototic Bid, a member of the Bcl-2 protein family, which is then translocated to the mitochondria. Bid oligomerizes with Bcl-2 proteins, Bax and Bak, leading to cytochrome c release (Korsmeyer et al., 2000; Schlossmacher et al., 2011). Cytochrome c a key participant in the mitochondrial electron transport chain responsible for ATP synthesis and its release into the cytoplasm induces the formation of the apoptosome complex, comprised of apoptotic protease activating factor (Apaf-1), cyctochrome c, and procaspase-9 (Acehan et al., 2002; Adams et al., 1998). A conformational change in the apoptosome activates caspase-9, which in turn, activates caspase-3 to induce apoptosis (Porter et al., 1999).

1.3 Significance

GCs are involved in almost every psychological, cellular and molecular mechanism of the human organism and are vital to the preservation of stress-related homeostasis through their regulation of metabolic and immune function. In a healthy organism, GCs terminate excessive inflammatory responses by inducing immune cell apoptosis (Distelhorst, 2002). The effects of GCs are mediated by the GC receptor (GR). Previous studies have demonstrated GC binding to GR causes the latter to activate and enter the nucleus, where it acts as a transcriptional regulator to promote apoptosis (i.e., GC-induced apoptosis). However, recent data have shown that activated GR can trigger faster apoptosis through protein-protein interaction in the intrinsic apoptotic pathway (Revollo et al., 2009).

We have previously shown that the LPS-stimulated splenocytes of SDR-treated mice are less sensitive to the anti-inflammatory effects of GCs (Avitsur et al., 2000).

Importantly, GC insensitive cells have been shown to traffic to other sites of the periphery and to regions of the central nervous system associated with threat appraisal, fear, and anxiety, where they play a pivotal role in propagating inflammation (Wohleb et al., 2011). Although the pathways associated with GC-induced apoptosis in normal cells have been extensively studied, the mechanism by which cells of stressed organisms develop resistance to GC-induced apoptosis has not yet been elucidated. We hypothesized that the abrogation of GC-induced apoptosis within the splenocytes of SDR-treated mice is a result of caspase-3 dysregulation, due to its key role inapoptosis. Interestingly, we report that activity and mRNA expression is elevated in SDR mice, suggesting that insensitivity to GC-induced apoptosis develops through a mechanism that does not include capsase-3.

2. Materials and Methods

2.1 Animals

Six to eight week-old male C57BL/6 and 12 month-old CD-1 (retired breeders) mice were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6 mice were housed three per cage in 11.5 in x 7.5 in x 6 in polypropylene cages (CD-1 mice were singly housed). Animals were allowed to acclimate to their surroundings for 7 days prior to experimental procedures. Animal housing rooms were maintained at 21 °C under a 12 h light/dark cycle (lights turned on at 0600) with *ad libitum* access to water and rodent chow. Experiments were conducted in accordance with a protocol approved by the Institutional Laboratory Animal Care and Use Committee at the Ohio State University).

2.2 Social disruption stress

The SDR paradigm has been described to a greater extent in previous publications from our laboratory (Avitsur et al., 2001; Bailey et al., 2007; Stark et al., 2001). Briefly, a CD-1 intruder mouse was placed into a cage of established C57BL/6 mice cohorts (2-3 per cage) for six consecutive nights for 2 h (17:00-19:00). Intruder mice were retired breeder mice that had previously shown aggressive behavior. If an intruder mouse did not initiate an attack within the first 5 min or was defeated by any of the resident mice, it was replaced by a new intruder. To ensure the resident mice were displaying submissive behavior each cycle of SDR, they were observed for wounding and submissive behavior, such as upright posture that exposed the abdominal region, fleeing, and crouching. Each cage was exposed to a different intruder on consecutive nights and the health of the resident mice was closely monitored. Severely injured or moribund mice were removed from the study. Home cage control (HCC) mice were housed in a separate room, and thus, isolated from visual, olfactory, and auditory stressor cues. Approximately 12 h after the last SDR cycle, SDR-treated and HCC mice were euthanized by CO_2 asphyxiation and spleens and blood were harvested for further analyses.

2.3 Blood and spleen processing

After CO_2 asphyxiation, blood was immediately collected via cardiac puncture with EDTA lined 1 ml syringes. Whole blood was centrifuged and serum was collected to determine IL-6 concentration. Spleens from experimental and control mice were aseptically removed, weighed, and mechanically disrupted in 5 ml of ice-cold Hanks-

balanced salt solution (HBSS) using a Model 80 Biomaster Lab System Stomacher (Seward, Riverview, FL). The resulting single cell suspensions were washed with additional ice-cold HBSS and pelleted. Red blood cells were lysed with 2 ml of room temperature RBC lysis buffer (8.8 g NH_4Cl, 1.0 g $KHCO_3$, 0.038 g EDTA, 1000 ml distilled H_2O) for 4 min followed by HBSS + 10% heat-inactivated fetal bovine serum (FBS) to stop the lysis reaction. The cell pellets were washed again, filtered through a 70 µm pore nylon filter, and resuspended (2.5 x 10^6 cells/ml) in 5 ml RPMI 1640 supplemented with 0.075% $NaHCO_3$, 10 mM HEPES buffer, 100 U penicillin G/ml, 100 µg streptomycin sulfate/ml, 1.5 mM L-glutamine, 0.00035% 2-mercaptoethanol, and 10% heat-inacativated FBS. Cell counts were obtained using a Z2 Coulter Counter (Beckman-Coulter, Brea, CA). All solutions were kept on ice during processing.

2.4 Culture conditions

The resistance of cells to inhibition by glucocorticoids was performed as previously described (Stark et al., 2001). Briefly, aliquots from every cell suspension (2 x 10^5 cells/50 µl) were stimulated with 1 µg/ml lipopolysaccharide (LPS; Sigma) and treated with physiological (0.005 µM) to pharmacological (5 µM) concentrations of corticosterone. Corticosterone was obtained commercially (Sigma) and diluted in supplemented RPMI 1640 and 0.2% ethanol. Cell suspensions were added in triplicate to flat-bottom 96-well plates (100 µl/well) and were incubated at 37 °C and 5% CO_2. After 45 h, the cell viability assay was performed. The remainders from each cell suspension (1.2 x 10^7 cells/ml) were also stimulated with 1 µg/ml and treated with 0.005-5 µM corticosterone. The cell suspensions were then added in duplicate to flat-

bottom 6-well plates (3 ml/well) and were incubated at 37 °C and 5% CO_2. After 18 h, cells from these plates were pelleted and used for RNA extraction and the Caspase-3 Colorimetric Assay while supernatants were frozen in -80 °C until assayed for IL-6.

2.5 Cell Viability Assay

To assess cell viability, a CellTiter 96 Aqueous non-radioactive proliferation assay was purchased from Promega (Madison, WI). The tetrazolium substrate solution was prepared according to the manufacturer's instructions, and 20 µl were added to each well of the 96-well plates. Metabolically active cells convert this substrate to formazan, producing a brown precipitate. The plates were incubated at 37 °C and 5% CO_2 for 3 h and the resulting color changes were quantified through optical density readings at 490 nm on an ELISA plate reader. To account for differences in background activity of cells, the mean optical density (OD) of the three RPMI wells for a given treatment was subtracted from each of the corresponding LPS-stimulated values. The results for each group are given as a percentage of LPS-stimulated proliferation with no corticosterone present.

2.6 RNA isolation and real-time PCR

RNA was isolated from LPS-stimulated splenocytes that were corticosterone-treated for 18h using an RNeasy Mini Kit (Qiagen, Gaithersburg, MD). RNA concentration was measured by spectrophotometry (Implen, Westlake Village, CA) and RNA was reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Quantitative PCR was

performed using a Taqman Gene Expression Assay to observe for potential changes in expression of CASP3 and CASP9. Expression was analyzed using an ABI Prism 7000 Sequence Detection System (Applied Biosystems; Foster City, CA) using the $2^{-\Delta\Delta}$ Ct method with normalization glyceraldehyde-3 phosphate dehydrogenase (GAPDH).

2.7 Caspase-3 colorimetric assay

LPS-stimulated splenocytes that were corticosterone-treated for 18h were used to measure caspase-3 activity using a Caspase-3 colorimetric assay (Genscript, Piscataway, NJ) as per manufacturer's instructions. Briefly, 10 µl dithiothreitol (DTT) + 5 µl phenylmethanesulfonylfluoride (PMSF) were added to 1 ml of provided lysis buffer. 50 µl of the prepared lysis buffer were added to each sample (5×10^6 cells/sample). Samples were incubated on ice for 40 min and centrifuged (10,000 rpm, 4 °C for 1 min). The supernatant was then collected and a Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) was used to quantify protein concentration. 150 µg of protein from each sample was added to a flat-bottom 96-well plate in triplicate and 50 µl 2X reaction buffer supplemented with 0.5 µl DTT and 0.25 µl PMSF was added to each well. Plates were incubated at 37 °C for 4 h immediately after the addition of 5 µl of the provided caspase-3 substrate solution. The resulting color changes were quantified through optical density readings at 400 nm on an ELISA plate reader.

2.8 IL-6 ELISA

IL-6 ELISAs were performed from supernatant gathered from LPS-stimulated splenocytes that were corticosterone-treated for 18h and from serum extracted from

whole blood. The ELISA was performed according to manufacturer's instructions (BD Biosciences, San Diego, CA). Briefly, plates were coated with 100 µl of anti-mouse IL-6 monoclonal antibody and incubated at 4 °C overnight. The antibody was then blocked with PBS/10% FPS and incubated for 1 h at room temperature. Following washes with PSB/Tween, 100 µl of sample/standard was added and the plates were incubated for 2 h at room temperature. The plates were aspirated and washed with PBS/Tween again and 100 µl of detector solution (biotinylated anti-mouse IL-6 monoclonal antibody and SAv-HRP reagent) was added to each well. The plates were incubated at room temperature for 1 h before being washed and again. 100 µl of substrate solution (tetramethylbenzidine and hydrogen peroxide) was then added to each well and incubated for 30 min in the dark. The reaction was stopped by the addition of 50 µl of 2N H_2SO_4 and an ELISA plate reader was used to measure optical density at 450 nm with a 570 nm correction.

2.9 Statistical Analysis

Duplicate or triplicate wells containing cells from the same mouse were averaged prior to calculating the group average. All data are presented as means ± the standard errors. One- or two-way ANOVA from http://easycalculation.com/statistics/one-way-anova.php was used to analyze significant main effects. Results were considered significant if $p \leq 0.05$.

3. Results

3.1 Increased Spleen-Body Weight Proportion in SDR-treated Mice

Before caspase-3 within the context of glucocorticoid-induced apoptosis could be studied, it was necessary to first confirm that the SDR effect was successfully established in the stressed mice. In our previous studies using the SDR model, four outcome measures were established to confirm successful SDR effect; splenomegaly, increased corticosterone resistance, increased circulating IL-6, and increased IL-6 production by GC-resistant cells (Avitsur et al., 2000; Stark et al., 2001; Stark et al., 2002). The first aim was to confirm the splenomegaly commonly associated with SDR-treated mice (Avitsur et al., 2000). Following euthanasia by CO_2 asphyxiation, mice body weight was determined. Spleens were then excised and weighed. Spleen-body weight proportion was found to be significantly higher in SDR-treated mice, confirming our previous findings (Fig. 1).

3.2 Increased Corticosterone Resistance in SDR Splenocytes

To determine if the splenocytes from stressed animals showed impaired responsiveness to glucocorticoid-induced apoptosis, splenocyte suspensions (2×10^5 cells/50 µl) from home cage control (HCC) and SDR mice were LPS-stimulated (1 µg/ml) and cultured with corticosterone concentrations ranging from physiological (0.005 µM) to pharmacological (5 µM). After 45 h of incubation, cell viability was measured with a CellTiter 96 aqueous nonradioactive proliferation assay. As shown in Fig. 2, splenocytes from SDR mice demonstrated increased resistance to apoptosis by corticosterone, which is another previously established marker of successful SDR effect (Stark et al., 2001).

3.3 IL- 6 Levels in Serum from SDR-treated Mice Are Elevated

As a third method of SDR confirmation, systemic levels of IL-6 were measured in blood serum. Immediately following CO_2 asphyxiation, whole blood samples were obtained from HCC and SDR-treated mice via cardiac puncture. Serum was then extracted from the blood and ELISA analysis was used to quantify IL-6 levels. Studies have previously shown plasma IL-6 is significantly low in non-stressed mice and significantly elevated in mice subjected to successful SDR (Stark et al., 2002). IL-6 in SDR serum was more than twice that of HCC IL-6, again suggesting that SDR effect was successfully established (Fig. 3).

3.4 IL-6 Concentration in SDR Cell Culture Supernatant Is Increased

As a final confirmation of SDR effect, ELISA analysis was used to examine whether SDR altered the IL-6 levels in the spleen. Spleens were harvested and aseptically homogenized into splenocyte suspensions. Cell suspensions were then LPS-stimulated and treated with either 0.1 μM or 0.5 μM coricosterone for 18 h. ELISA analysis of the supernatants from each sample showed SDR splenocytes produced elevated levels of IL-6 when LPS-stimulated and subjected to corticosterone (Fig. 4).

3.5 Caspase-3 Activity Is Elevated in SDR Splenocytes

Once a successful SDR effect was confirmed, caspase-3 activity was measured using a Caspase-3 Colorimetric Assay. Splenocyte suspensions of every sample were LPS-stimulated and treated with either 0.1 μM or 0.5 μM corticosterone for 18 h. Cells were lysed and 150 μg of protein from each sample was used to measure caspase-3

activity. Interestingly, caspase-3 activity was determined to be higher in SDR splenocytes (Fig. 5).

3.6 CASP3 & CASP9 Expression Is Increased in SDR Splenocytes

To determine caspase-3 and -9 mRNA expression in splenocytes subjected to 0.1 μM and 0.5 μM corticosterone for 18h, quantitative PCR was performed. RNA was extracted and reverse transcribed to cDNA. CASP3 and CASP9 expression was measured with a Taqman Gene Expression Assay, with GAPDH as a housekeeping control. F6 shows capspase-3 mRNA is upregulated in SDR splenocytes, which supports the increased caspase-3 activity measured by the Caspase-3 Colorimetric Assay. Caspase-9 mRNA expression was also upregulated, albeit to a lesser extent, which additionally supports the previous results (Fig. 7).

4. Discussion

The SDR effect was successfully confirmed by the splenomegaly of SDR-treated mice (Fig. 1), and the cell viability assay, which showed splenocytes of SDR-treated mice were more resistant to the induction of apoptosis by corticosterone than HCC mice (Fig. 2). This indicates that GC-induced apoptosis was significantly weakened in SDR-treated mice. Also, it has been previously established that SDR-treated mice have significantly elevated levels of systemic IL-6 and that their splenocytes produced more IL-6 when LPS-stimulated (Stark et al., 2002). Thus, as an additional method of confirmation of SDR effect, IL-6 ELISAs of supernatant acquired from splenocytes and blood serum were performed. Both the supernatant and serum IL-6 ELISAs show

significantly higher IL-6 concentration in SDR-treated mice, confirming that the intended SDR effects were successfully established in the treated mice (Fig. 3 & 4).

After the SDR effect was successfully established, a caspase-3 enzymatic activity was measured, and showed a significant increase relative to activity present in SDR splenocytes than HCC (Fig. 5). Quantitative PCR supports the caspase-3 assay analysis. We showed that caspase-3 and caspase-9 mRNA expression is increased in SDR-treated mice when compared to HCC mice (Fig. 6 & 7).

The results did not support the stated hypothesis, which predicted that caspase-3 would be reduced in SDR-treated mice. The SDR paradigm caused insensitivity to GC-induced apoptosis. Thus, it was either expected for caspase-3 gene expression to be suppressed or for caspase-3 protein to have reduced activity. Increased caspase-9 mRNA expression in SDR also supports the idea that caspase-3 is properly activated, as caspase-9 regulates caspase-3 activity. Thus, the findings did not show that a lack of adequate caspase-3 production or activity in socially stressed animals was responsible for the insensitivity of their splenocytes to GC-induced apoptosis.

The results of the study are promising, albeit fairly limited, in characterizing the mechanism by which glucocorticoid-induced apoptosis develops in socially stressed mice. One possible future direction would be to study caspase-3 structure in SDR to investigate if a change in structural configuration has altered caspase-3 functionality. If caspase-3 functionality is compromised it would prevent it from interacting with its downstream targets, such as DFF45/ICAD, whose activation allows DFF40/CAD to begin internucleosomal DNA degredation, and Bcl-2 and Bcl-xL, which become

proapoptotic when activated (Gross et al., 1999). A second future direction would be to investigate the role of caspase-6 and -7 in GC-induced apoptosis. Although it is well known that caspase-3 is the key effector, it is also known that caspase-3 is not responsible for all of the downstream events in apoptosis (Slee et al., 2001). Thus, it is possible that caspase-6 or -7 dysregulation is responsible for the insensitivity to GC-induced apoptosis that is observed in socially stressed mice. Also, an interesting hypothesis is that apoptosis may be induced by Bcl-2 translocation to the lysosomes, where it allows the formation of pores causes membrane permeabilization (Zhao et al., 2001). Thus, a third possible direction would be to investigate Bcl-2, and its family members, changes in activity and translocation in response to SDR.

Additionally, it is now well known that caspase activation is not the only determinant of life and death in apoptosis (Blagosklonny et al., 2000; Kitanaka et al, 1999; Lockshin et al., 2000). For example, Bax-induced cell death occurs despite inhibition of caspase activation (Xiang et al., 1996). In fact, evidence now suggests that programmed cell death can occur in a completely caspase-independent manner (Borner et al., 1999; Boya et al., 2003). Both Apoptosis-inducing factor (AIF) and Endonuclease G are able to directly induce DNA fragmentation and chromatin condensation in a caspase-independent manner (Joza et al., 2001; Li et al., 2001). Thus, it is possible that caspase-3 is not a mediator of the apoptosis shown in this study. In order to further investigate this possibility, an additional direction for the study would be to completely inhibit caspase-3 activation in order to potentially reveal underlying caspase-independent apoptotic pathways.

In conclusion, the results demonstrated the insensitivity to GC-induced apoptosis in SDR is not a result of caspase-3 dysregulation. The mechanism of the downstream apoptotic steps is not fully understood. The majority of chemotherapeutic agents, such as GCs, induce the intrinsic apoptosis pathway, however the possibility of GC-induced cell death in a caspase-independent fashion may be worth pursuing further. Additional insight into the changes associated with insensitivity to GC-induced apoptosis may lead to better characterization of how GCs exert their effects and to the identification of novel targets through which tissue sensitivity to GCs can be regulated, decreasing patient susceptibility to pathology.

FIGURE 1

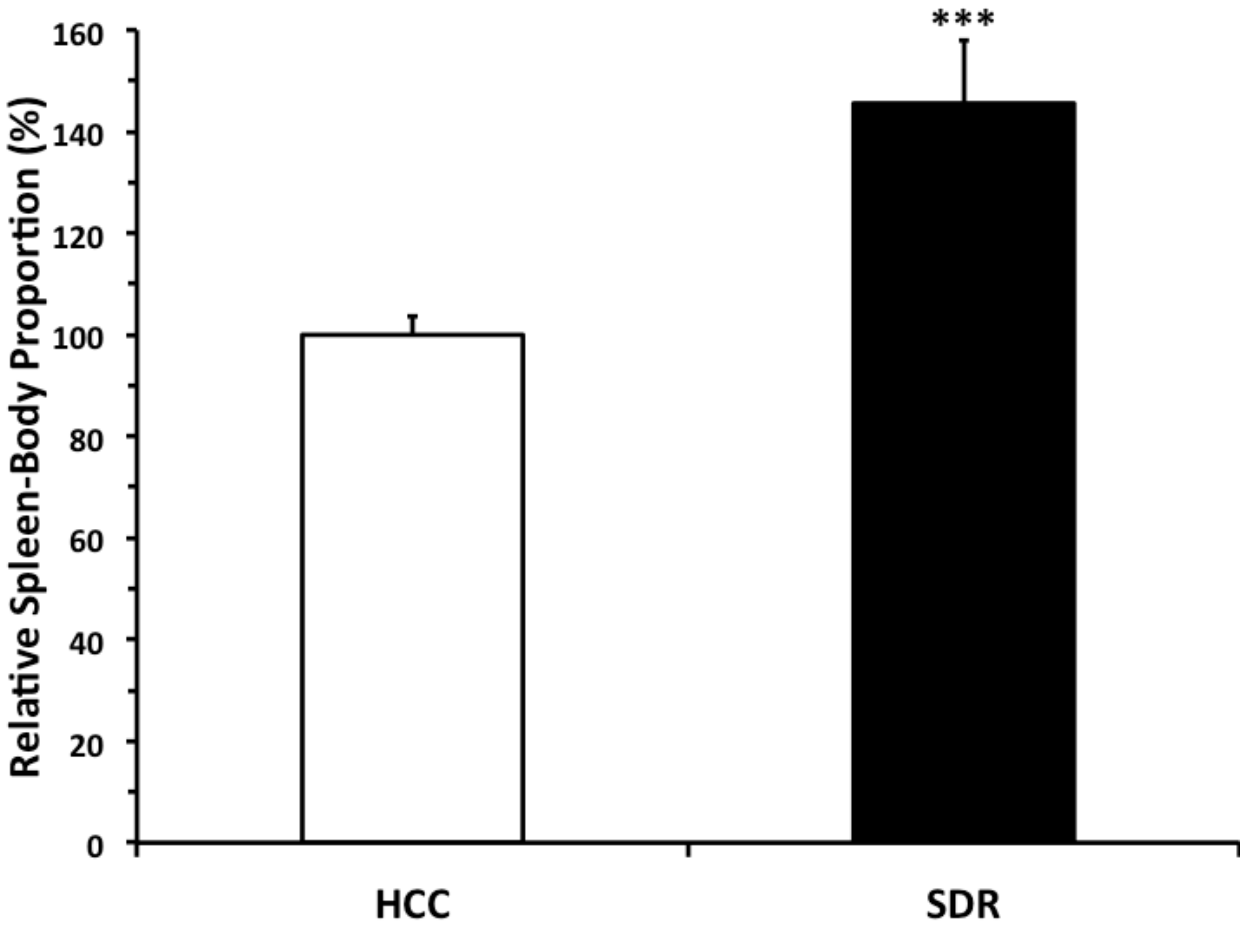

***
160
140
120
100
80
60
40
20
0
Relative Spleen-Body Proportion (%)
HCC
SDR

FIGURE 2

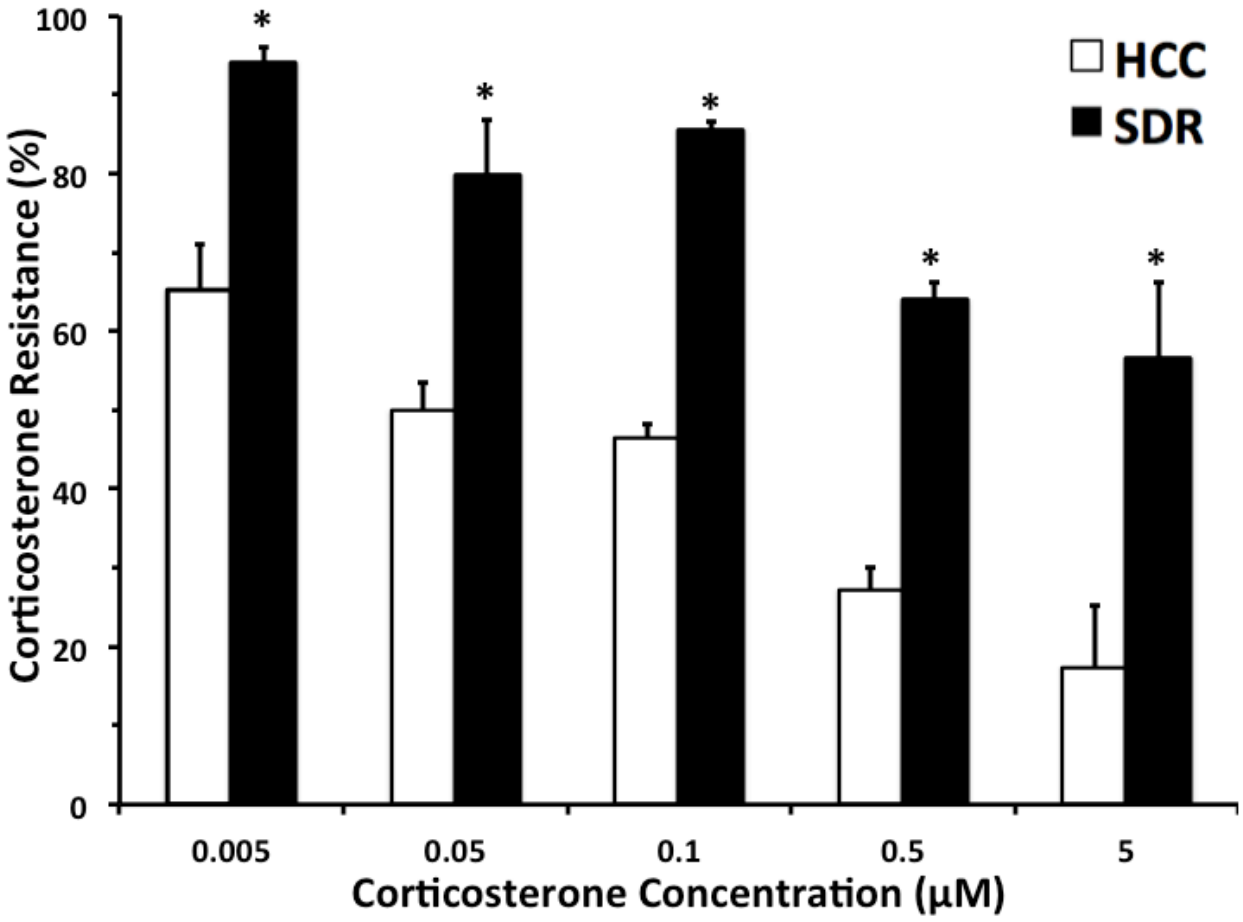

100
80
60
40
20
0
Corticosterone Resistance (%)
HCC
SDR
*
*
*
*
*
0.005
0.05
0.1
0.5
5
Corticosterone Concentration (µM)

FIGURE 3

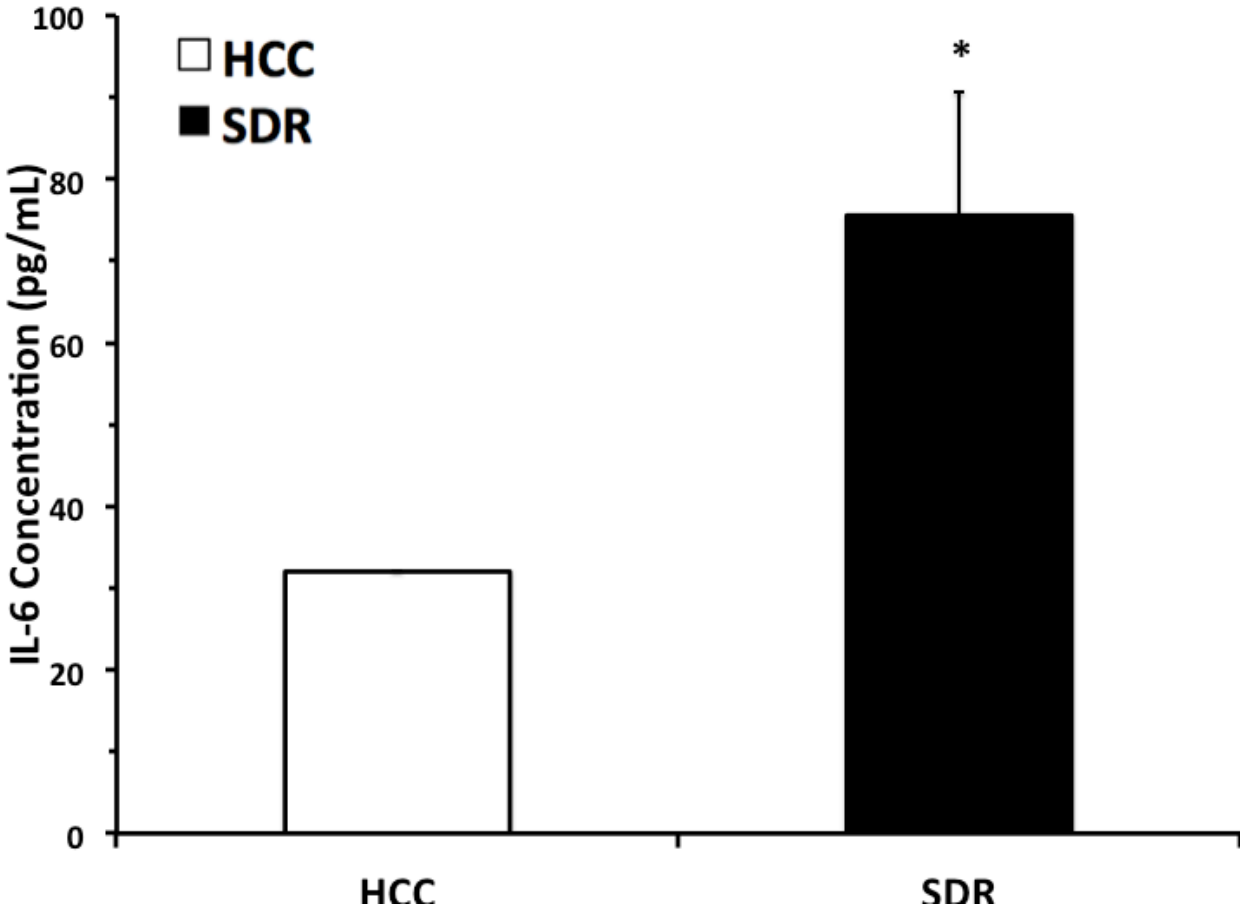


FIGURE 4

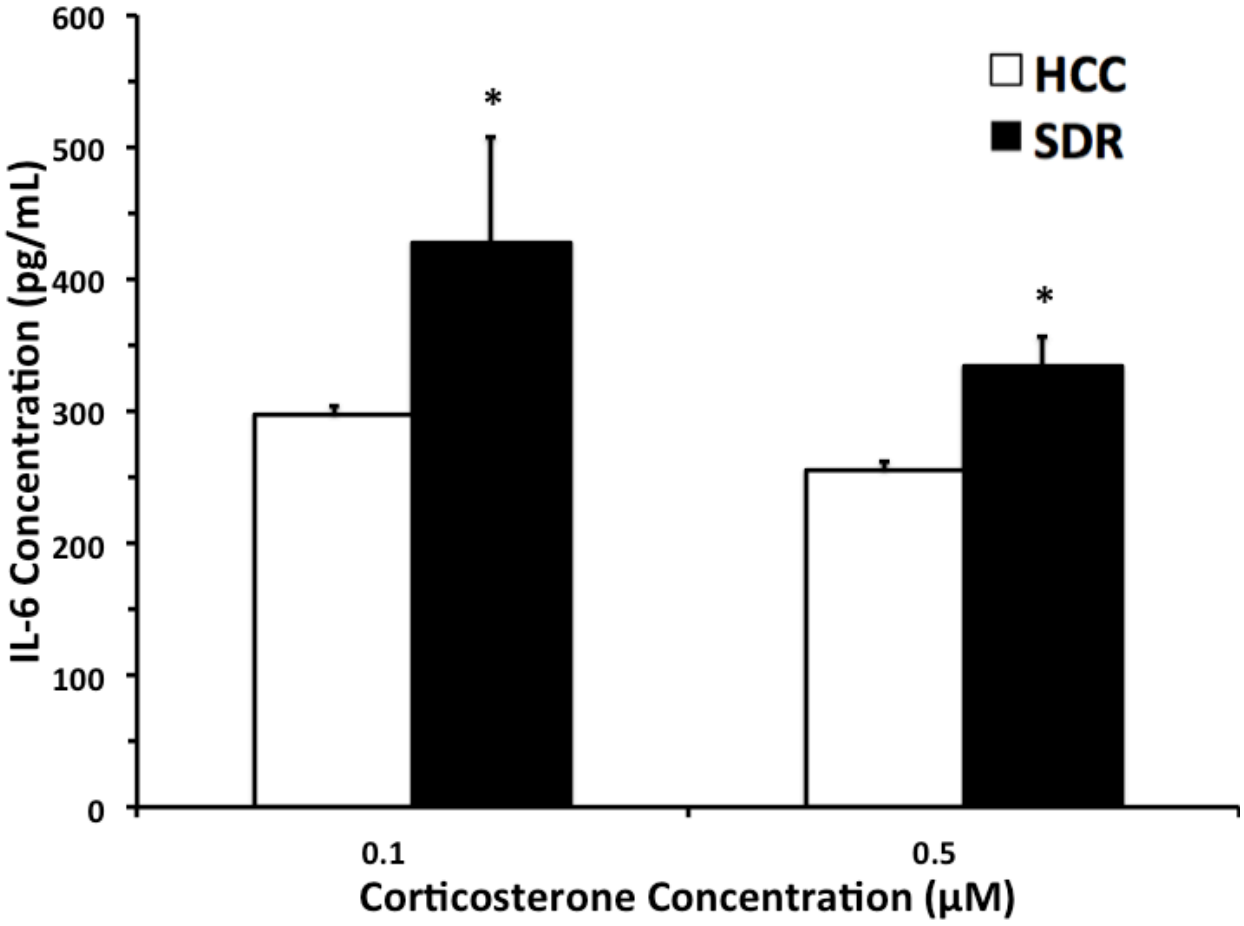

600
500
400
300
200
100
0
IL-6 Concentration (pg/mL)
HCC
SDR
*
*
0.1
0.5
Corticosterone Concentration (µM)

FIGURE 5

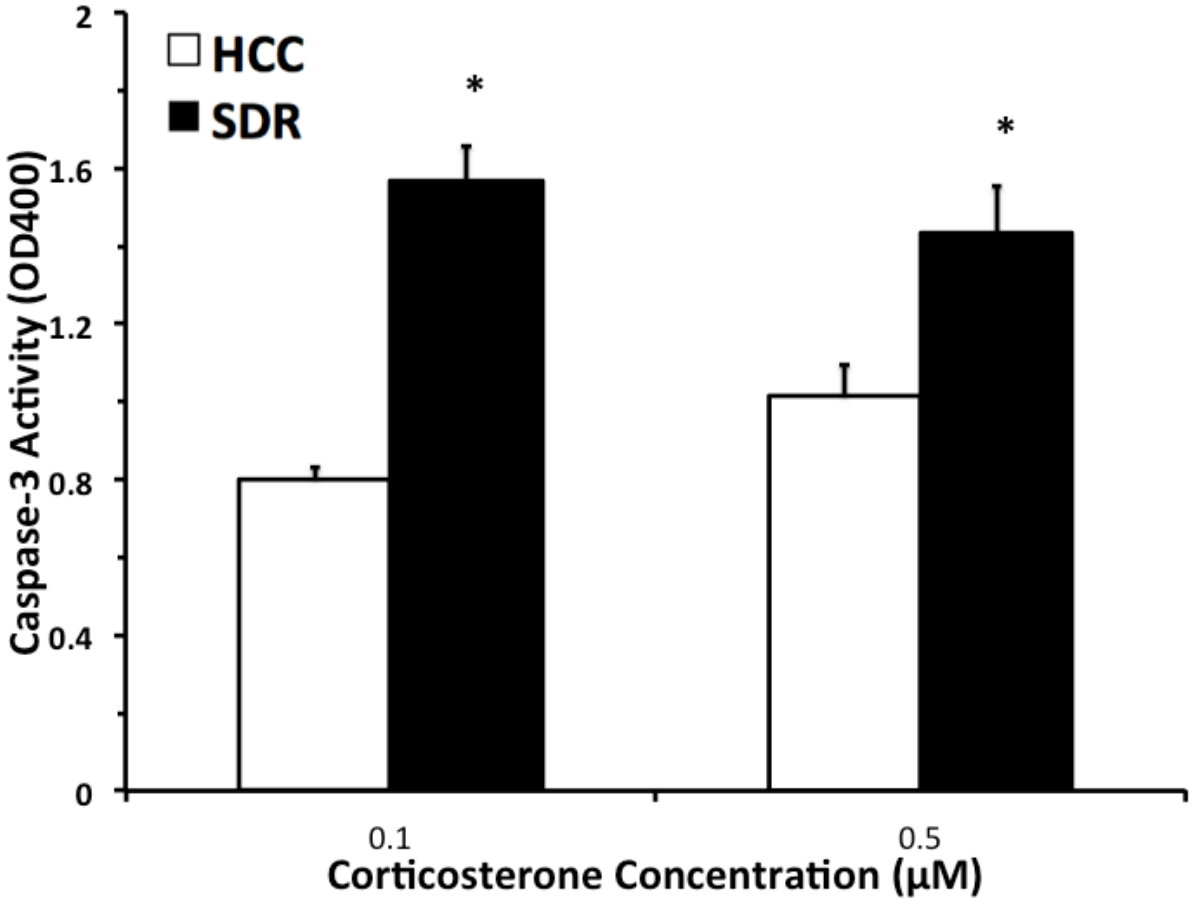
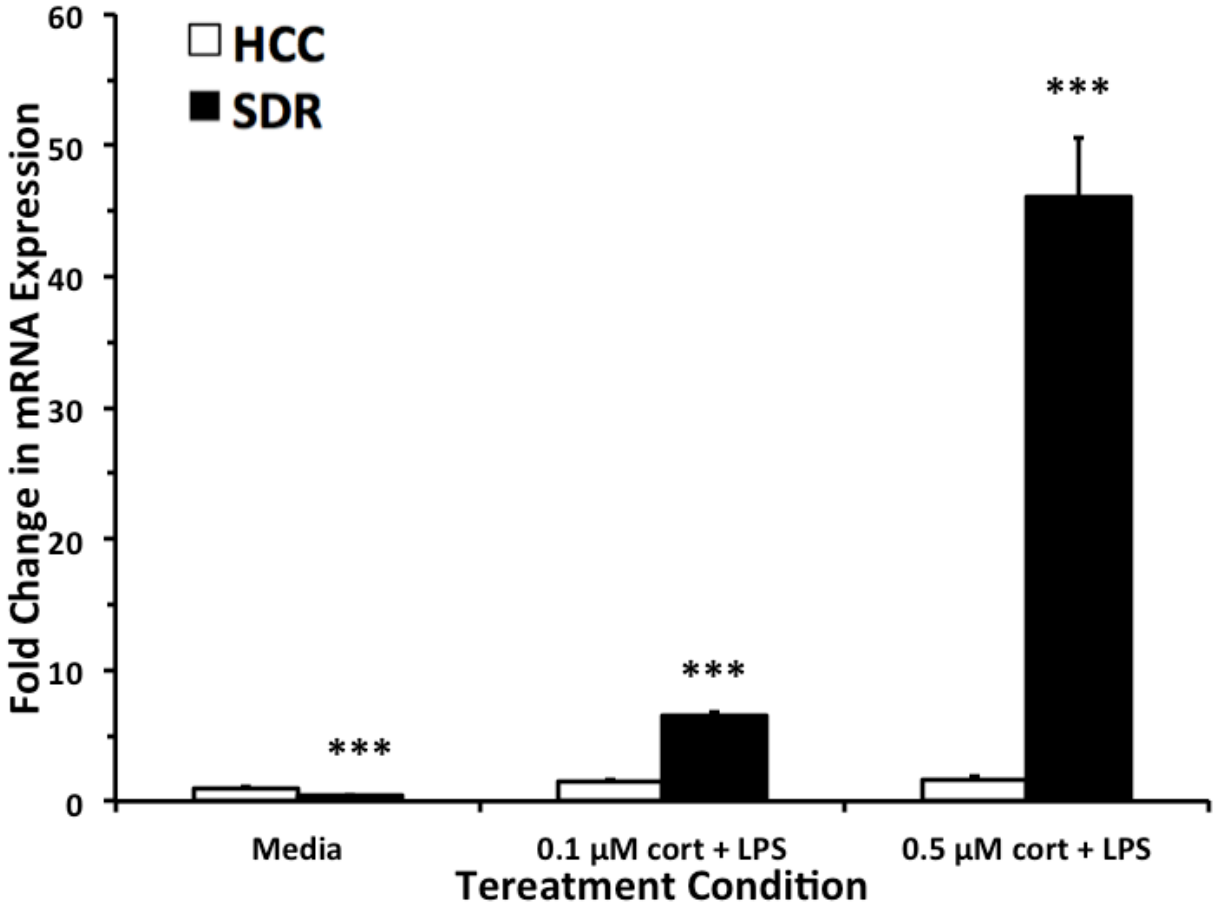

HCC
SDR
*
*
Caspase-3 Activity (OD400)
2
1.6
1.2
0.8
0.4
0
0.1
0.5
Corticosterone Concentration (μM)

FIGURE 6


HCC
SDR
Fold Change in mRNA Expression
60
50
40
30
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10
0
***
***
***
Media
0.1 µM cort + LPS
0.5 µM cort + LPS
Tereatment Condition

FIGURE 7

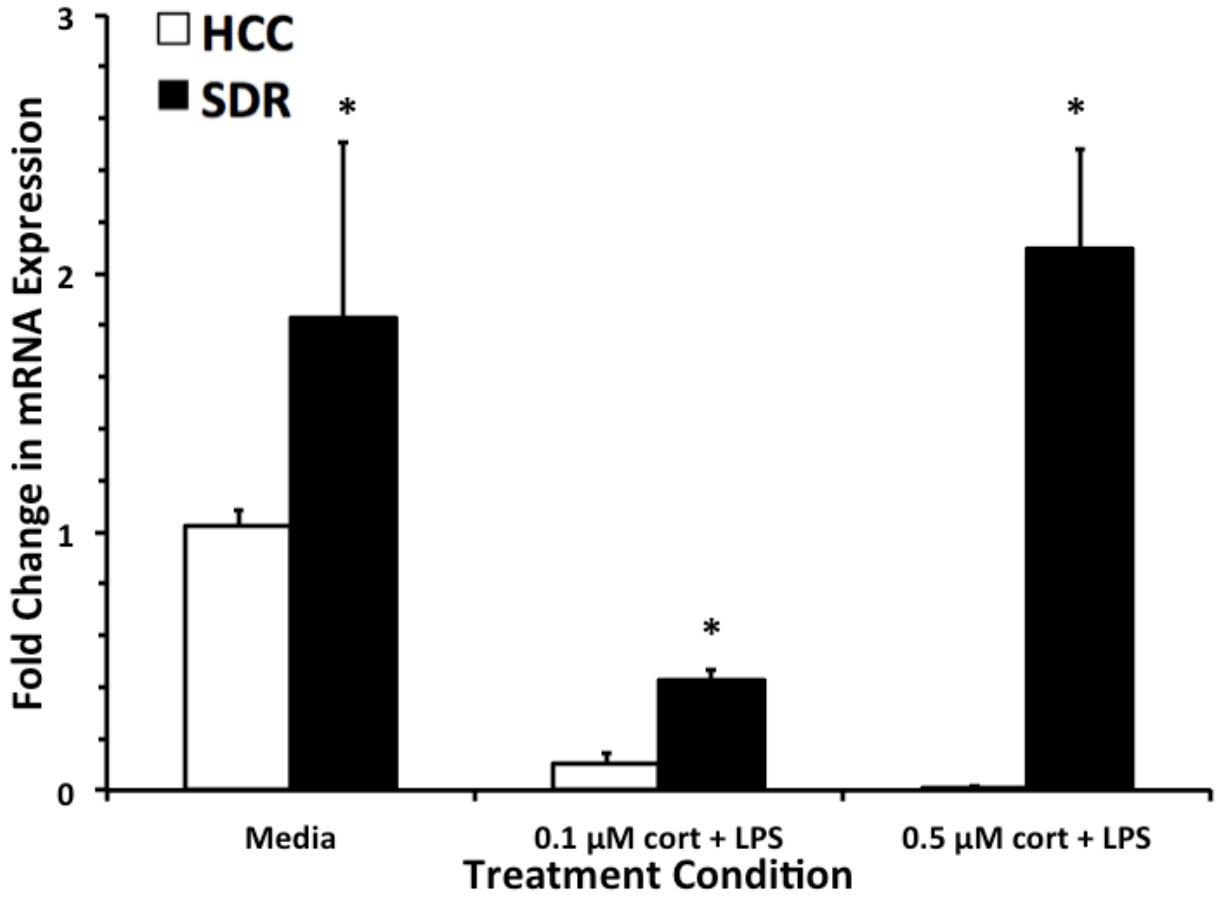
HCC
SDR
Fold Change in mRNA Expression
3
2
1
0
*
*
*
Media
0.1 µM cort + LPS
0.5 µM cort + LPS
Treatment Condition

FIGURE LEGENDS

Fig. 1 Increased Spleen-Body Weight Proportion in SDR-treated Mice. Six to eight week-old male C57BL/6 mice were subjected to social disruption stress for six consecutive nights. Approximately 12 h after the last SDR cycle, experimental and control mice were euthanized by CO_2 asphyxiation. Each mouse was weighed and spleens were harvested and also weighed. Bars represent the spleen-body proportion of SDR-treated mice relative to the HCC proportion. SDR spleen-body proportion is significantly higher than HCC. (mean+/- SEM, HCC n= 21, SDR n=25, *** p< 0.001)

Fig. 2 Increased Corticosterone Resistance in SDR Splenocytes. To test the resistance of cells to apoptosis by glucocorticoids, aliquots of splenocyte suspensions (2×10^5 cells/50 µl) were stimulated with 1 µg/ml LPS and treated with 0.005 - 5 µM corticosterone for 45 h. Cell viability was then measured with a plate reader. Bars represent the average of the biological replicates. SDR splenocytes are significantly more resistant to corticosterone than HCC. (mean+/- SEM , HCC n=6, SDR n=6, 3 mice per group, *p<0.05)

Fig. 3 IL- 6 Levels in Serum from SDR-treated Mice Are Elevated. After the mice were euthanized, blood was collected via cardiac puncture. Serum was collected from the collected whole blood. An ELISA was used to quantify IL-6 levels in serum. IL-6 concentration in SDR serum is more than twice the HCC serum IL-6 concentration. (mean+/- SEM, HCC n=3, SDR n=3, 3 mice per group, *p<0.05)

Fig. 4 IL-6 Concentration in SDR Cell Culture Supernatant Is Increased. LPS-stimulated splenocytes (1.2 x 10^7 cells/ml) were treated with corticosterone (0.005- 5 μM) for 18 h. Supernatants from each sample were gathered after 18 h and an ELISA was used to quantify IL-6 concentration in the supernatants from each sample. SDR LPS-stimulated splenocytes produced more IL-6 than HCC. (mean+/- SEM, HCC n=4, SDR n=4, 3 mice per group, *p<0.05)

Fig. 5 Caspase-3 Activity Is Elevated in SDR Splenocytes. LPS-stimulated splenocytes that were corticosterone-treated for 18h were used to measure caspase-3 activity using a Caspase-3 Colorimetric Assay according to manufacturer's instructions. Caspase-3 has increased activity in SDR LPS-stimulated splenocytes subjected to corticosterone. (mean+/- SEM, HCC n=2, SDR n=2, 3 mice per group, 5 x 10^6 cells per group, *p<0.05)

Fig. 6 CASP3 Expression Is Increased in SDR Splenocytes. RNA was isolated from LPS-stimulated splenocytes that were corticosterone-treated for 18h. RNA was then reverse transcriped to cDNA and quantitative PCR was performed to measure CASP3 expression. Caspase-3 mRNA is upregulated in SDR LPS-stimulated splenocytes subjected to corticosterone. (mean+/- SEM, HCC media n=3, SDR media n=5, cort-treated n=1, 3 mice per group, duplicate wells, ***p<0.001)

Fig. 7 CASP9 Expression Is Increased in SDR Splenocytes. RNA was isolated from LPS-stimulated splenocytes that were corticosterone-treated for 18h. RNA was then reverse transcriped to cDNA and quantitative PCR was performed to measure CASP9 expression. Caspase-9 mRNA is upregulated in SDR LPS-stimulated splenocytes

subjected to corticosterone. (mean+/- SEM, HCC media n=3, SDR media n=5, cort-treated n=1, 3 mice per group, duplicate wells, *p<0.05)

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